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Investigation of DNA Interaction with Bis-Cationic Imidazolium Salts

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Abstract

Imidazolium salt compounds have shown promise *in vitro* against non-small cell lung cancer (NSCLC) lines, but the exact mechanism of activity these compounds is still unknown. Due to their planar aromatic structure, we hypothesized that our bis-cation imidazolium salt series could act through DNA intercalation similar to known intercalators ethidium bromide, acridine orange, and echinomycin. Here, we tested bis-cation imidazolium salts with linkers of 2, 4, 10, and 12 carbons for DNA intercalation via viscosity and fluorescent intercalator displacement (FID) assays. Despite some solubility issues that may have decreased results, we found that our butyl and dodecyl linked compounds had activity exceeded that of known intercalator, acridine orange. Results from the FID assay were less profound and none of the compounds were able to significantly displace ethidium bromide as an intercalator in a DNA solution. These findings indicate that bis-cation imidazolium salts likely do not have mechanistic activity against DNA, and future studies should evaluate other intracellular targets to determine the mechanism.

Introduction

In 2016, it is projected that 224,390 new cases of lung cancer will be diagnosed, 83% of which will be characterized as NSCLC.[1] Currently, the pharmaceutical drug cisplatin is utilized for a variety of cancer cell lines due to its activity, but its effectiveness is not without a cost. Use of cisplatin can lead to toxicity and associated adverse effects in patients.[2] The focus of the Youngs group is to synthesize imidazole compounds that can match the activity of cisplatin while reducing the adverse effects. Synthesis of our imidazolium salts have centered around the addition of aromatic planar groups, such as naphthalene, to the 1,3 positions on the imidazole ring.[3] The planar structure of these compounds led to the hypothesis that their activity could mimic the effects of known DNA intercalators such as ethidium bromide and acridine orange.[4] Activity of these imidazole compounds against NSCLC lines have shown promise in vitro, however, knowledge of their specific mechanism of action is limited.

The purpose of this project is to begin studies to investigate the mechanistic activity of our bis-cation series of imidazolium salts in cancer lines through viscosity and fluorescent intercalator displacement (FID) assays. Due to the planar aromatic nature of these compounds, we propose that DNA is a viable intracellular target to begin investigating. Motivation for the synthesis of double intercalation compounds such as our bis-cation series has stemmed from research on echinomycin, a known double intercalator.[5] Our bis-imidazolium cation series is composed of compounds containing 1-12 carbons existing between imidazolium rings bound with naphthalene groups (**Figure 1**). Based off of our knowledge of echinomycin, we hypothesize that our bis-cation series could possibly double intercalate DNA and the length of the linker between the imidazole groups will greatly effect when and how this intercalation will occur.

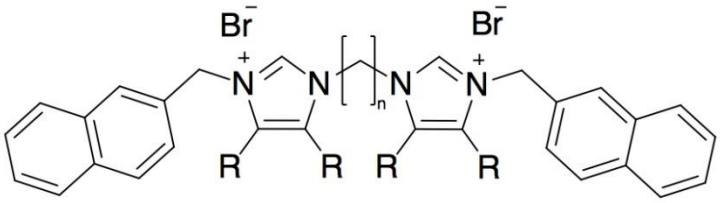

Figure 1. The bis-cationic imidazolium series that will be tested with 1-12 carbons existing as the n group and hydrogen as the R groups.

When intercalation occurs, base pairs surrounding the intercalation site will bend due to the strain placed on the DNA. To relieve this strain the DNA backbone will partially unwind, fractionally increasing the length of the DNA which will be detectable during a test of viscosity.[6] Our FID studies will rely on a titration of our compounds into a DNA solution intercalated with ethidium bromide. The pi-pi interaction between the planar, aromatic rings of ethidium bromide and the base pairs of DNA create a significant increase in fluorescence. Our hope is that our compounds will displace the ethidium bromide and intercalate the DNA represented by a detectable decrease in fluorescent intensity.[7] With combined results from viscosity and FID studies, we will be able to better understand how our

compounds interact with and intercalate DNA and how linker length may provide insight into how bis-cation species can be modified in the future.

Materials and Methods

Reagents

Bis-cation imidazolium salts with linkers of 2, 4, 10 and 12 were chosen for analysis. The compounds were synthesized by fellow undergraduate student Stephen Crabtree. Due to the compounds' varying degrees of solubility in water, dimethyl sulfoxide (DMSO, Fisher Scientific) was used at 10% volume for both viscosity and FID assays.

Calf thymus DNA (CT-DNA, Invitrogen) solutions that were used for both the viscosity and FID assay were created combining CT-DNA and tris-NaCl buffer (Buffer was created using Invitrogen UltraPure™ Distilled Water with Fisher Scientific tris at 5 mM and NaCl at 50 mM). A DNA concentration within the range of 225-250 μM for the viscosity assay and 115-135 μM for the FID assay were desired. For the viscosity DNA stock, 560 μL of CT-DNA with an initial concentration of 10 mg/mL was added to 70 mL of tris-NaCl buffer. For the FID stock solution, these values were only 30 mL of buffer and 123 μL of CT-DNA. These solutions were mixed thoroughly with swirling and a sample from each was tested for concentration using an ultraviolet-visible spectrophotometer (Cary 100 Bio UV-Visible Spectrophotometer). The absorbance values collected from the peaks of the readings were divided by the extinction coefficient of the CT-DNA, 6600 M^{-1} cm^{-1} to obtain the concentration of DNA in solution.[6] If the target concentration for the assay was not obtained after the initial additions adjustments to the volume of CT-DNA or buffer were made accordingly until the solution was brought into the appropriate range as verified by the UV-Vis.

Weighing compounds for solutions

Solutions for each of the compounds were created so that the concentration of compound was increased 10 μM per 8 μL addition for viscosity and 3.33 μM per 2 μL addition for FID. This meant that our 1 mL compound solutions would need to be 10 mM solutions for viscosity and 5 mM for FID. Using

the varying molecular masses of the compounds being tested with the known total volumes and concentration values, the masses needed for each of the compounds were calculated as seen in **Table 1**.

Viscosity Assay

The methods for the viscosity assay were adapted from Fu et al.[7] A 10 mL viscometer was positioned in a water bath at 30 ˚C. Temperature changes were closely monitored using a glass thermometer as they could drastically alter the flow through times of the DNA solution. The viscometer was anchored in the water bath so that the entire sample was submerged during testing.

Eight mL of tris-NaCl buffer was pipetted into the large arm of the viscometer and drawn up through the smaller arm using a 3-valve rubber pipette bulb. The solution was drawn up until the bulb on the smaller arm of was completely filled. Removing the bulb, gravity began drawing the meniscus of the solution down towards the white line directly above the bulb on the small arm of the viscometer. Flow time of the solution was defined by the time it took the meniscus of the solution to move from the white line above the bulb to the white line directly below the bulb. The trial was repeated 3 times and an average was calculated. After the buffer runs were completed, the solution was disposed of and 8 mL of CT-DNA solution was pipetted into the viscometer and tested using the same procedure as used for the buffer.

After the completion of three similar runs of the CT-DNA in buffer, the first addition of compound in DMSO and water was added to the viscometer and the existing CT-DNA solution. The bis-cation compound was prepared for addition by dissolving the pre-weighed amount first in 100 μL of DMSO and brought up to volume with 900 μL of DNase-RNase free water. Eight microliter additions that would increase the concentration of compound in solution by 10 μM were chosen. Using a pipette, the compound solution was deposited on the inner wall of the narrower arm approximately one inch from the top of the arm. The three valve pipette bulb was used to draw the solution from the bulb on the wider arm up to where the compound was added to bring the compound into solution. This was repeated 3-4 times to ensure that the compound was completely removed from the wall and brought into solution.

Mixing of the solution occurred via a standard rubber pipette bulb which was used to force the solution from the thin arm through the loop and into the large bulb on the thicker arm. The exerted pressure supplied to the bulb forced air through the viscometer and the solution in the bulb resulting in a bubbling effect. The bubbling should be induced 2-3 times for a length of 1-2 seconds. Once the solution is bubbled through, the three valve bulb can be used to draw up the solution for the first of the three trials. These steps were followed for the remaining 6 additions bringing the final concentration of the compound in solution to 70 μM after the final addition.

FID Assay

The protocol utilized for the FID assay was adapted from Boger et al.[8] and conducted on a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer. The spectroflourometer was turned on and allowed to warm for 30 minutes prior to the beginning of the assay. The intensity of the lamp alone and that of a cuvette filled with nanopure water were tested at the beginning of each day's trial as controls to ensure that the instrument was working properly and the data collected would be representative of the compound tested. Using the CT-DNA stock solution, 2.997 mL of DNA and 3 μL ethidium bromide were pipetted into a clean, 4 mL cuvette. The contents were mixed by pipette and the cuvette was placed in the sample holder of the spectrofluorometer. The sample incubated for two minutes in the dark prior to measuring the emission spectrum of the sample from 465 nm to 765 nm with an excitation peak at 510 nm. Following the run, data was extracted from the reading and inputted into an excel spreadsheet for ease of access.

The bis-cation compound was prepared for addition by dissolving the pre-weighed amount first in 100 μL of DMSO and brought up to volume with 900 μL of DNase-RNase free water. Additions consisted of 2 μL increments from 2-30 μL, 10 μL increments from 30-100 μL, and 100 μL increments from 100-600 μL. During each addition, the given volume was pipetted directly into the cuvette, and mixed by pipette. Once again, 2 minutes was given as an incubation period before analysis on the fluorometer.

Results and Discussion

Weighing compounds for solutions

The compounds for each of the viscosity and FID trials were weighed out as seen in **Table 1** below.

Table 1. The mass of each of the compounds needed to make a 1mL, 10 mM solution for viscosity assay and a 1mL, 5 mM solution for the FID assay.

Mass of Compounds Needed for Solution			
Viscosity Assay		FID Assay	
# of linker	Mass (g)	# of linker	Mass (g)
2	0.0060	2	0.003
4	0.0063	4	0.00315
10	0.0072	10	0.0036
12	0.0074	12	0.0037

Viscosity Assay

Six separate trials were conducted for the viscosity assay; a vehicle control of 10% DMSO in water, the known DNA intercalator acradine orange, and the four compounds with ethyl, butyl, decyl, and dodecyl linkers. After normalizing data to compensate for small variations in the temperature between trials, the trials were graphed as seen in **Figure 2** below.

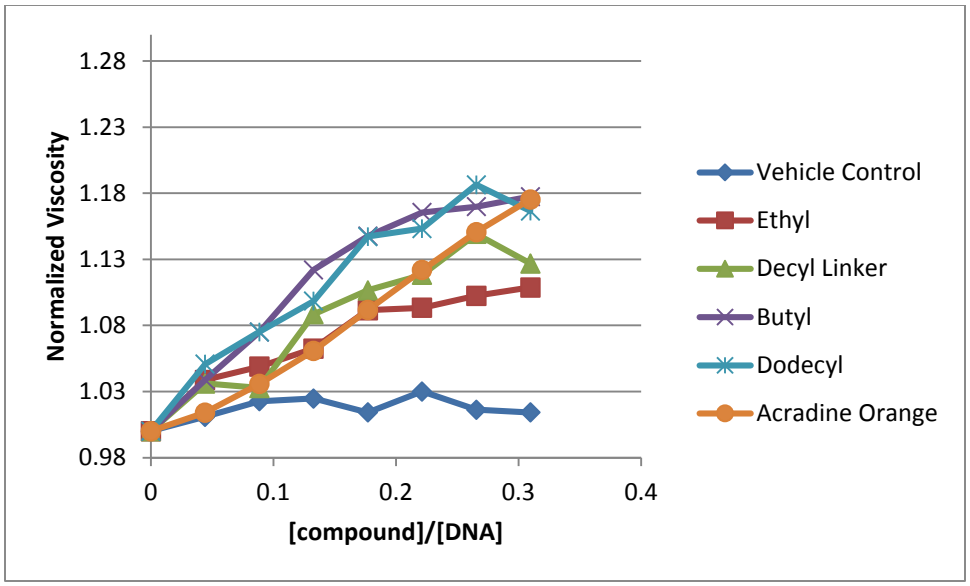

Figure 2. The normalized viscosity of each of the six trials graphed against the ratio of compound divided by the DNA concentration.

The viscosity for the interactions of the DNA solution with both the dodecyl linker and the butyl linker showed activity that indicated more DNA intercalation that our reference solution, acradine orange. Although not as significant as the dodecyl and butyl linkers, the ethyl and decyl linkers showed moderate activity, but not to the extent of acridine orange. As aforementioned, the bis-cation imadazolium salt series has varying solubility in water, and despite the addition of 10% DMSO, the compounds had issues with solubility that increased with linker length. The ethyl and butyl compounds dissolved completely in solution for the duration of the trials, but the decyl and dodecyl compounds crashed out of solution several times between additions to the sample within the viscometer. Despite the use of a Fisher Scientific Vortex to mix the contents, solid compound remained. This insolubility likely resulted in inconsistent increases in concentration, suggesting the activity of the two compounds may be misrepresented by their data. The concentration increases were likely reduced by the precipitated solids implying that the activity of these compounds may have been more significant than Figure 2 indicates.

Solubility issues with our compounds present a large issue should there be a desire to move forward into cell tests based on the results of this study. DMSO served as an agent to increase compound solubility in this study, but cannot be used in vivo due several unfavorable interactions within cells including inducing apoptosis in the CNS of mice[9] and damaging mitochondrial integrity in astrocytes.[10] The oligosaccharide cyclodextrin may provide the answer as it has shown promise in drug delivery applications using its hydrophilic exterior and lipophilic interior to carry drugs through the bloodstream to their eventual targets.[10] The Youngs group has experimented with the use of cyclodextrin for drug delivery systems, but use of the sugar with the bis-cation series has yet to be investigated using this viscosity assay.

FID Assay

The intensity values collected for the FID assays on the spectrofluorometer were normalized against the intensity value recorded for 0 microliters compound added for each given day of testing.[8] Data normalization ensures that the data collected from the given compounds are representative of their interactions with DNA and ethidium bromide and not altered by the decreasing power of the spectrofluorometer's lamp or other changes that could occur with the device between the trials. Analysis of data was completed for each of the four compounds, a vehicle control of 10% DMSO in water, and Netropsin, a reference compound known to displace ethidium bromide and intercalate with DNA.[8] **Figure 3** visualizes the drops in intensity for each of the 6 trials for 2 µL additions from 0-30 µL. All four of the compounds showed equally insignificant drops in intensity that did not rival that of the known DNA intercalator and ethidium bromide displacer, netropsin. This trend continued as the volumes increased to 10 µL and eventually to100 µL additions suggesting that the compounds do not have significant enough intercalation with CT-DNA to displace ethidium bromide.

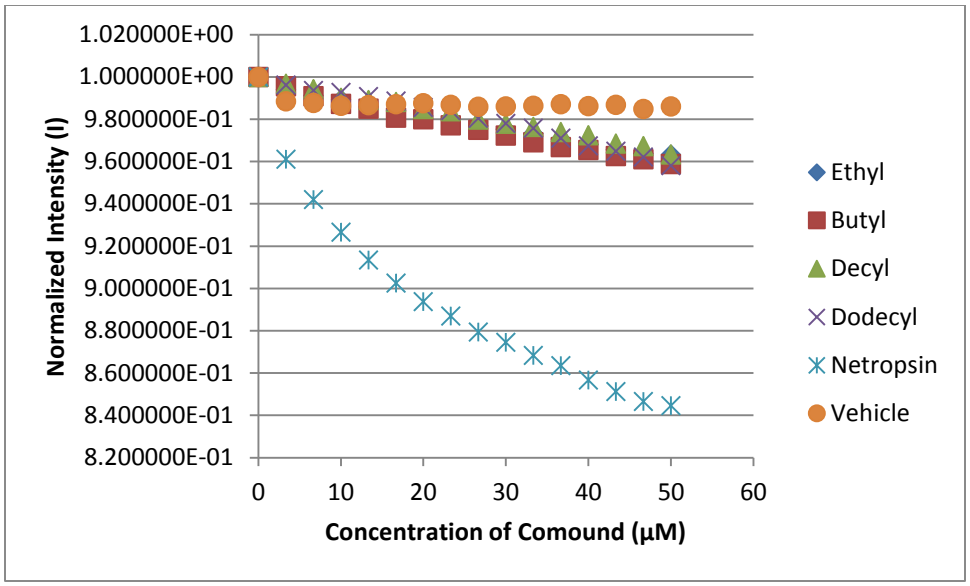

Figure 3. The FID assay data for the four compounds, 10% DMSO vehicle control, and netropsin. Intensity measured on the spectrofluorometer was normalized each day and graphed against the concentration of the compound added for each individual trial.

The FID assay did produce some interesting findings that revealed an interaction that occurs between the bis-cation imidazolium salts with longer linkers and our CT-DNA solution. Both the dodecyl and decyl linked compounds were found to precipitate the CT-DNA when volumes of the compounds exceeded 100 µL. Precipitation of the DNA in solution resulted in a color change from a translucent, red-pink color provided from the diluted ethidium bromide to a foggy, orange color. These qualitative changes were accompanied by significant quantitative changes as well as intensity values in the foggy solutions were recorded 2-3 factors lower than previously measured. This finding is extremely problematic as the precipitation of DNA would prohibit the compounds from realistically being used *in vivo* unless they are able to interact with a different intracellular target.

Conclusions

In this study, bis-cation imidazolium salts with varying length linkers were tested to see if their interaction with CT-DNA might reveal a mechanistic action that could be utilized to treat NSCLC. Two assays that test DNA intercalation, a viscosity assay and a fluorescence intercalation displacement assay,

were completed with inconsistent results. The viscosity assay tested compounds against both a vehicle control and the known DNA intercalator acridine orange. Despite solubility issues with the longer linked compounds that could have resulted in the addition of smaller concentrations, data showed an increase in viscosity. The data collected indicated that the butyl and dodecyl linkers added greater strain to the DNA and increased viscosity more than acridine orange and that the ethyl and decyl linkers showed moderate activity, but not as significant as acridine orange. Problems with solubility could have led to significant error in the exact values recorded, however, general trends in run-through times indicate all of the compounds increased viscosity. The inclusion of the vehicle control proves that the viscosity was not increasing solely because of the 10% DMSO solution being added, but was a product of the compounds themselves. Despite the positive results of this assay, the results do not give us any information about the placement of interaction within the major or minor grooves of DNA helices. Future studies of DNA intercalation with bis-cation compounds could reveal how the increases in viscosity are occurring.

The FID assay results did not indicate the same promise that the viscosity study did for the compounds. Salts with ethyl and butyl linkers showed little to no intercalation displacement of ethidium bromide and appeared insignificant in comparison to the positive control, netropsin. The dodecyl and decyl linkers showed similar inactivity at the lower concentrations before precipitating out the DNA once compound volume neared 100 μL. DNA precipitation is a seriously problematic byproduct for compounds designed to be anti-tumor agents, so it is unlikely that compounds above a certain number of carbons could be used for future studies unless their mechanism of action leads them to an alternative intracellular target. It is possible that a compound with a linker between 5-9 carbons could have the increased intercalation displacement of netropsin without precipitating out like the decyl and dodecyl compounds. With the inconsistencies of our bis-cation series in relation to DNA interaction, the next logical step for the Youngs group will be to investigate others mechanisms of action for our bis-cation series.

Acknowledgments

I would like to thank a few individuals who contributed to the success of this Honors Research Project. First, I would like to thank Marie Southerland who gave both her time and talents to train me on the techniques that I utilized to collect data and then was a major help during the editing process of this paper. I would also like to thank my fellow undergraduates Taylor Mattioli and Stephen Crabtree. Stephen was responsible for synthesizing all of the compounds that I used during the study and I knew that I could count on him to produce clean, pure compounds that would be reliable for testing. Taylor was equally as valuable in the completion of the experiment as she trained me on the FID assay and answered all of the questions I had about testing the compounds. Finally, I would like to thank my readers for this project, Dr. Youngs, Dr. Tessier, and Dr. Shriver. I truly appreciate the time that each of them has given to reading my drafts and providing constructive criticism that lead to this final product.

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Safety Appendix

Laboratory safety training and instruction was provided by Marie Southerland, the graduate student overseeing this project. Standard Personal Protection Equipment (PPE) was required for all aspects of both of the assays conducted and students were required to wear nitrile gloves and safety glasses at all times. Specific care was required for several of the reagents used in the study, especially the known DNA intercalators ethidium bromide, acridine orange, and netropsin. Special waste containers for liquid samples that contained ethidium bromide or netropsin as well as solid samples that contained these reagents at some point (such as tips) were kept in a fume hood in sealed glass containers. To limit handling by an undergraduate student, Marie was responsible for the addition of ethidium bromide into the CT-DNA samples during the FID assay trials to avoid any unnecessary risk and prevent spills. After the completion of these trials, safety protocols were followed that involved disposing the samples and several washes of the cuvette containing them in the aforementioned waste containers before disposing any washes down the sink. The less potent DNA intercalator acridine orange was handled with similar care to ethidium bromide taking specific precaution when dealing with concentrated stock solutions. Due to its reduced toxicity, samples containing the reagent were discarded in normal aqueous, non-halogenated waste after experiments.

Dimethyl sulfoxide (DMSO) was used during the study to increase the solubility of our compounds in water. Although DMSO is generally regarded as safe in small volumes, the effects of our bis-cation imidazolium salts on human cell lines are generally unknown given the experimental nature of this study. For this reason, special containers similar to those for ethidium bromide waste were kept in a fume hood for all aqueous solutions containing imidazolium salts without any ethidium bromide. These containers were stored to be collected by the UA Health and Safety Department who ultimately decide the final neutralization and disposal methods.

In addition to the specific disposal protocols followed for this study, general health and safety practices were followed. No food or drinks were allowed in the laboratory during the conducting of experiments and proper clothing such as closed toed shoes and pants covering the legs were required. If

students did come in contact with chemicals during the course of the study, eye-wash and chemical shower station locations were highlighted by Marie Southerland. Spilling of chemicals of any kind, splashing of reagents into eyes, nose, or mouth of an individual or cuts resulting from broken glassware were to be reported to Marie or Dr. Youngs and depending on the severity of the situation, the UA Health and Safety Department.